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Identification of *O*-Aminoacetophenone as a Flavour Compound in Stale Dry Milk

In our efforts to improve the keeping quality of dry milk products, attention has been directed towards isolating and identifying flavour compounds present in stale products. This communication deals with the isolation of a compound with a 'grape-like' flavour, identified as *o*-aminoacetophenone. Preliminary work on the solvent extraction of stale non-fat dry milk indicated that a strong flavour fraction characterized as potato-like or cocoa-like could be extracted with hexane. The odour was materially altered by adding a strong acid but could be regenerated by adding alkali. Crude concentrates were prepared by hydrochloric acid extraction of hexane extracts of stale dry milk powder. The alkali-liberated flavour fraction was back extracted in small volumes of hexane which were then gas chromatographed on an 'Apiezon *L*' column. A flavour fraction, 'grape-like' in character, was detected with the nose at the tail pipe of the instrument. The odour emergence coincided with a characteristic peak on the recorder. This flavour material was collected in a cold trap attached to the outlet of the column and was shown to contain nitrogen by the method of Feigl and Amaral[1]. The material collected from numerous successive chromatograms was analysed in a mass spectrometer. The results indicated that the sample was 'Apiezon' grease contaminated with a small amount of a compound with a parent peak at mass 135. Infra-red analysis indicated the possible presence of carbonyl oxygen and aromatic structure. The observations were consistent with aminoacetophenone. Examination of the authentic isomers revealed that *o*-aminoacetophenone had an odour identical with the natural fraction collected from the gas chromatograph. Further evidence supporting the identity of the isolated compound as *o*-aminoacetophenone is presented.

One-hundred-and-fifty g of 3-year-old stale non-fat dry milk was added to 600 ml. of hexane[2] in a Hamilton Beach model 30 drink mixer. (Reference to certain products or companies does not imply an endorsement by the Department over others not mentioned.) The contents were mixed thoroughly for 5 min after which 15 ml. of distilled water were added followed by an additional mixing time of 5 min. The slurry was transferred to a thimble and extracted continuously for 2 h in a Soxhlet extractor. The hexane extract was transferred to a 1-l. separatory funnel and extracted 3 times, each with 30 ml. of distilled water. The hexane solution was then extracted with 30 ml. of a 10 per cent aqueous hydrochloric acid solution. The acid solution was made alkaline with solid potassium carbonate and back extracted with 100 ml. of hexane. The hexane solution

Table 1. Gas Chromatographic Retention Data of Authentic and Isolated *O*-Aminoacetophenone

Sample	'Apiezon *L*' (125°) Retention time (min)	'Apiezon *L*' (125°) Relative retention	Diethylene glycol adipate (148°) Retention time (min)	Diethylene glycol adipate (148°) Relative retention
Authentic *o*-aminoacetophenone	11·4	0·39	43·4	4·72
Isolated *o*-aminoacetophenone	11·4	0·39	43·2	4·70
Methyl laurate	29·5	1·00	9·2	1·00

was dried with sodium sulphate, filtered, and evaporated under vacuum at room temperature to approximately 0·1 ml. of solution. Portions of this concentrate as well as authentic samples of *o*-aminoacetophenone and methyl laurate were gas chromatographed on two different columns and the results are recorded in Table 1. One column was a 6 ft. × ¼ in. stainless steel tube packed with 10 per cent 'Apiezon *L*' on 60–80 mesh silaned 'Celite'. The other column, of the same dimensions, contained 10 per cent diethylene glycol adipate on 'Anakrom *ABS*'. The columns were operated under 20 lb./in.^{2} argon inlet pressure with an ionization detector.

Further evidence for the presence of *o*-aminoacetophenone in stale dry milk was obtained as its 2,4-dinitrophenylhydrazone. For comparison, the authentic derivative was prepared in the following manner: 0·5 g of 2,4-dinitrophenylhydrazine was dissolved in 12 ml. of 30 per cent sulphuric acid. To the reagent was added 3 ml. of 30 per cent sulphuric acid containing 0·36 g of *o*-aminoacetophenone. The solution was allowed to stand overnight. Ten ml. of water were added to the reaction flask and the precipitate, recovered on a fritted glass funnel, was washed with 20 ml. of distilled water. The precipitate was transferred to an Erlenmeyer flask, 20 ml. of water added and the mixture made alkaline with solid potassium carbonate. The precipitate recovered by filtration was washed with warm ethanol and recrystallized from chloroform (m.p. 247°–248° C; elemental analysis: calculated: carbon 53·33 per cent; hydrogen 4·12 per cent; found: carbon 53·23 per cent; hydrogen 4·25 per cent; maximum absorption in chloroform 398 mμ; molar extinction coefficient 20,250). This derivative was submitted to each of the chromatographic steps described below for the experimental sample in order to establish the criteria for the isolation and identification.

A hexane solution of the concentrate from 450 g of the stale dry milk was reacted with 2,4-dinitrophenylhydrazine according to a slightly modified version of the column procedure of Schwartz and Parks[3]. The reaction column contained a mixture of 200 mg of 2,4-dinitrophenylhydrazine, 1·5 ml. of phosphoric acid (85 per cent), and 0·5 ml. of water impregnated on 2 g of analytical grade 'Celite'. Following passage of the hexane solution of the unknown through the reaction column, 50 ml. of hexane

containing 0·5 g of heptadecanone-2 were passed through the column to remove the majority of excess 2,4-dinitrophenylhydrazine reagent. The reaction column was then washed with carbonyl-free hexane[3] until the effluent was free of colour. The column packing was then stripped with 30 ml. of redistilled methanol. Sixty ml. water were added to the methanol extract and the solution made slightly alkaline with solid potassium carbonate and extracted with 50 ml. chloroform. The chloroform solution was dried with sodium sulphate and evaporated to dryness on a steam bath with the aid of a stream of nitrogen. The reaction residue in 10 ml. of chloroform was added to a chromatographic column containing 4 g of 'Celite 545' and 2 g of 'Magnesia 2665' prepared in chloroform according to the procedure of Schwartz *et al.*[4]. The residual 2,4-dinitrophenylhydrazine (green colour on column) was eluted with 100 ml. of chloroform. Following the chloroform wash, 0·5 per cent methanol in chloroform was added to the column and the *o*-aminoacetophenone DNP-hydrazone (lavender colour on column) was eluted in the second 50 ml. fraction. The eluate was evaporated to dryness and subjected to column partition chromatography on a 10-g 'Celite' column using 2 per cent ethyl acetate in methyl *cyclo*hexane saturated with acetonitrile as the mobile phase and acetonitrile as the immobile phase according to Corbin's procedure[5]. The unknown DNP-hydrazone recovered from the column had a maximum absorption at 390 mμ in chloroform in contrast to a maximum absorption at 398 mμ for the authentic *o*-aminoacetophenone DNP-hydrazone. Paper chromatography of the isolated DNP-hydrazone by the method of Sundt and Winter[6], however, revealed a trace contaminant moving slightly above the isolated *o*-aminoacetophenone DNP-hydrazone which could not be removed by further fractionation on magnesia or the partition system of Corbin. In order to confirm the identity, the unknown DNP-hydrazone was regenerated with sulphuric acid[7], and after making the regeneration mixture alkaline with solid potassium carbonate it was extracted with hexane. Evaporation of the hexane yielded a residue exhibiting the proper odour and gas chromatographic properties of *o*-aminoacetophenone. Table 2 contains threshold volumes and R_F values of *o*-aminoacetophenone DNP-hydrazone.

The flavour threshold of *o*-aminoacetophenone was determined in fresh pasteurized skim-milk to be 0·4 part per billion (Table 3) by the method of Patton and Josephson[8]. The quantity isolated from the very stale powder was 8–10 times the threshold quantity as determined by spectrophotometry of the 2,4-dinitrophenylhydrazone. Presumptive evidence of its occurrence in evaporated milk has been obtained by observing the characteristic odour at the proper emergence time from gas chromatography of evaporated milk extracts.

Work is in progress to elucidate the flavour significance of this compound in typically stale dairy products. By

Table 2. PARTITION CHROMATOGRAPHIC DATA OF AUTHENTIC AND ISOLATED *O*-AMINOACETOPHENONE DNP-HYDRAZONES

O-Aminoacetophenone DNP-hydrazone	Threshold volume*†	R_F‡
Authentic	27·0	0·11
Isolated	28·6	0·11

* Method of Corbin—column 2.
† Millilitres of mobile phase per gram of 'Celite'
‡ Method of Sundt and Winter.

Table 3. FLAVOUR THRESHOLD DATA FOR *O*-AMINOACETOPHONONE IN SKIM-MILK*

	Concentration (p.p.b.)					
	0	0·001	0·01	0·1	1·0	5·0
Positive responses	7	11	12	9	34	36
% Positive response	19	31	33	25	94	100

* Seven tasters evaluating a total of 36 samples of each concentration.

itself, the compound does not simulate stale flavour when added to fresh fluid milk ; however, when added to reconstituted slightly stale dry milk it intensifies the stale flavour. It appears that the compound is an important component of a mixture of compounds which collectively are responsible for typical stale milk flavour.

The source and mode of formation of *o*-aminoacetophenone are unknown at this time. Potential precursors to this compound in dairy products include tryptophan, indican (indoxyl sulphate), and kynurenine. It has been observed that the presence of *o*-aminoacetophenone in dairy products tends to accompany the Maillard reaction.

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